

Normalized levels of red blood cells expressing phosphatidylserine, their microparticles, and activated platelets in young patients with β -thalassemia following bone marrow transplantation

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Abstract Bone marrow transplantation (BMT) serves as the only curative treatment for patients with β -thalassemia major; however, hemostatic changes have been observed in these BMT patients. Aggregability of thalassemic red blood cells (RBCs) and increased red blood cell-derived microparticles (RMPs) expressing phosphatidylserine (PS) are thought to participate in thromboembolic events by initially triggering platelet activation. To our knowledge, there has been no report providing quantitation of these circulating PS-expressing RBCs and RMPs in young β -thalassemia patients after BMT. Whole blood from each subject was fluorescently labeled to detect RBC markers (CD235a) and annexin-V together with the known number TruCount™ beads. PS-expressing RBCs, RMPs, and activated platelets were identified by flow cytometry. In our randomized study, we found the

decreased levels of three aforementioned factors compared to levels in patients receiving regular blood transfusion (RT). This study showed that BMT in β -thalassemia patients decreases the levels of circulating PS-expressing RBCs, their MPs, and procoagulant platelets when compared to patients who received RT. Normalized levels of these coagulation markers may provide the supportive evidence of the effectiveness of BMT for curing thalassemia.

Keywords Activated platelets · Beta-thalassemia · Bone marrow transplantation · Microparticles · Phosphatidylserine-expressing red blood cells

Introduction

β -Thalassemia is a group of chronic hemolytic anemias caused by the absence or insufficient production of β -globin chains that form tetramers with α -globin chains. Patients with β -thalassemia major receive regular blood transfusions (RTs) to maintain their hemoglobin levels and mitigate anemic condition; however, this treatment is not curative [1, 2]. At present, bone marrow transplantation (BMT) serves as the only cure for patients with certain hematological diseases, including β -thalassemia major, homozygous β -thalassemia, and hemoglobin E/ β -thalassemia. Young patients are offered treatment with BMT if matched donor stem cells are available [3–5].

Hemostatic changes and thrombotic events are frequently observed in patients undergoing BMT. As a consequence of abnormal hemoglobin synthesis in β -thalassemia, the α -globin chains precipitate and form the inclusion bodies within red blood cells (RBCs) causing oxidative stress and perturbing

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the normal asymmetric composition of the lipid membrane. Phosphatidylserine (PS), which is normally localized in the inner membrane leaflet, switches to the outer membrane and this can be detected by probing with annexin-V in a calcium-dependent manner [6–9]. PS-externalizing RBCs play an important role as inducers of a hypercoagulable state in thalassemia by triggering chronic platelet activation [9]. In addition, PS can be recognized and bound by coagulation factors, especially tissue factor and factor VII, facilitating further proteolysis, activation of factor X, and generation of thrombin [9]. These PS-expressing RBCs are reported to be increased in hemolytic anemias such as sickle cell disease [10, 11], paroxysmal nocturnal hemoglobinuria [12], and thalassemia [13].

Furthermore, microparticles (MPs) (smaller than 1 μm in diameter) shed from RBCs, called red blood cell-derived microparticles (RMPs), have been documented [14–16]. RMPs also bear PS on their surface and are described as the second most frequent of circulating MPs after platelet-derived MP [17]. In thalassemia, RMPs are elevated in patients who have undergone splenectomy compared those in non-splenectomized patients and might play a role in activating platelets [14, 18]. To our knowledge, RMP levels in patients post-BMT have never been studied. Here, we report the detection in whole blood of hypercoagulation parameters, including PS-exposing RBCs, RMPs, and procoagulant platelet levels, in young β -thalassemic patients who received RT or have undergone BMT.

Subjects, materials, and methods

Subjects

Blood samples were obtained from β -thalassemic patients prior to receiving regular blood transfusion and those following BMT in which engraftment was achieved as revealed by full chimeras of donor cells. The post-BMT duration ranged from 2 months to 2 years. All BMT patients received hematopoietic stem cells from donors who were normal and had no background of being heterozygotes or carriers of thalassemia except for one patient who received BMT from a haploidentical donor with thalassemia trait. Age- and sex-matched healthy volunteers were also recruited for this study. After giving written consent, blood samples were collected in K_3EDTA and buffered sodium citrate tubes, carried on ice, and processed within 4 h. This study was approved by the Ethics Committee of the Faculty of Medicine Ramathibodi Hospital (MURA2014/553).

Complete blood count

K_3EDTA blood samples were mixed and passed through hematological analyzer (Sysmex Xi-800, Kobe, Japan).

Whole blood staining

To detect PS-expressing RBCs and RMPs, 10 μl of citrate blood were diluted in phosphate-buffered saline (PBS) at 1:10 to reduce spontaneous platelet activation and shedding of platelet-derived microparticles. Then, 10 μl of diluted blood were added to Trucount™ tubes (BD Biosciences, San Jose) containing a known number of counting beads. Ninety microliters of a mixture of saturated monoclonal antibodies comprised of phycoerythrin (PE)-conjugated annexin-V and fluorescein isothiocyanate (FITC)-conjugated anti-CD235a monoclonal antibody (mAb) in 0.2- μm pre-filtered annexin-V binding buffer was added into Trucount tubes (all antibodies were from BD Biosciences), and samples were incubated for 15 min in the dark at room temperature. Three hundred microliters of filtered annexin-V binding buffer were added into each sample tube to bring the final volume to 400 μl .

For determination of activated platelets, 10 μl of diluted blood were incubated in another polystyrene tube with allophycocyanin (APC)-conjugated anti-human CD41a mAb and either PE-conjugated anti-human CD62P mAb or anti-human CD142 mAb. Ninety microliters of PBS were added and samples were incubated for 15 min in the dark at room temperature. Then, 1% paraformaldehyde was added to reach a final volume of 350 μl before flow cytometric analysis using flow cytometer FACS CantoII, (BD Biosciences). Isotype controls were also used.

FACS analysis

All parameters, including forward scatter (FSC), side scatter (SSC), and fluorescence detection channels, were set up in logarithmic scale. Polystyrene beads (Spherotech, Illinois) of 1- μm diameter were used to set the gate for the MP region. The annexin-V binding buffer was run first to identify the noise background. Next, the sample tubes were run and acquisition was stopped when 1500 events of Trucount beads were collected. RBCs that were probed with annexin-V were identified as PS-expressing RBCs, and annexin-V⁺ MPs bearing CD235a were called RMPs. The absolute numbers of PS-exposing RBCs were calculated from the percentage of PS-exposing RBCs multiplied by the RBC count, whereas the total numbers of RMPs were calculated from the formulae below. The flow cytometric gating strategies are shown in Fig. 1.

Absolute number of MP

$$= \frac{(\text{Ann-V}^+ \text{ events} \times \text{number of beads in each tube} \times \text{dilution factor})}{\text{Number of collected beads} \times \text{total volume of sample}}$$

The population of platelets expressing activation marker (CD62P) and tissue factor (CD142) was investigated by

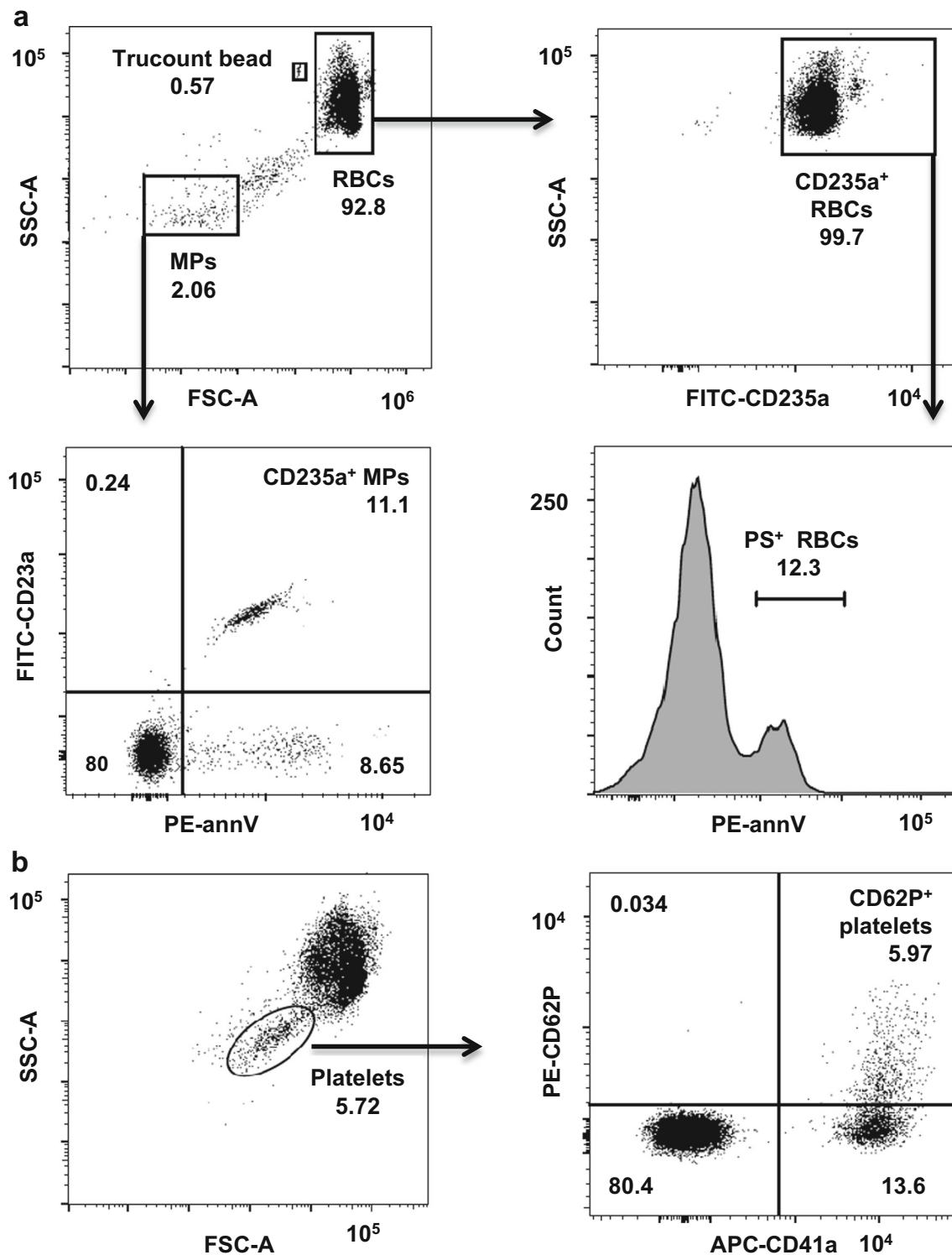


Fig. 1 Representative flow cytometric gating strategies for identifying **a** PS-exposing RBCs, RMPs, and **b** activated platelets. RBC and RMP populations were identified by their CD235a⁺ and annexin-V⁺ expression using plots with logarithmic scales of SSC/CD235a and CD235a/annexin-V, respectively. RBCs exposing PS were detected by their

annexin-V⁺ staining while RMPs were identified by their annexin-V⁺/CD235a⁺ expression. The population of platelets expressing activation marker and tissue factor were identified by the positivity to CD41a/CD62P or CD142 if used

Table 1 Hematological parameters of normal individuals and two thalassemic patient groups

Parameters	Normal subjects	β -Thalassemic patients	
		Regular transfusion	Bone marrow transplantation
Age (year)	12.4 \pm 3.9	13.2 \pm 4.4	12.3 \pm 5.3
Sex (M/F)	12/4	9/13	20/26
WBC count ($10^3/\mu\text{l}$)	6.1 \pm 0.4	11.8 \pm 3.6*	6.3 \pm 0.3 ^{###}
RBC count ($10^6/\mu\text{l}$)	4.9 \pm 0.1	3.6 \pm 0.1***	5.1 \pm 0.1 ^{###}
Hb (g/dL)	13.6 \pm 0.3	8.3 \pm 0.3***	13.0 \pm 0.4 ^{###}
Hct (%)	41 \pm 0.8	25 \pm 0.9***	36 \pm 1.1* ^{###}
MCV (fL)	84 \pm 0.9	68 \pm 1.6***	72 \pm 1.5***
MCH (pg)	28 \pm 0.4	23 \pm 0.5***	26 \pm 0.7 ^{###}
MCHC (%)	33 \pm 0.2	33 \pm 0.4	36 \pm 0.2***, ^{###}
RDW (%)	15.3 \pm 0.5	24.9 \pm 1.1***	14.9 \pm 0.4 ^{###}
PLT count ($10^3/\mu\text{l}$)	262 \pm 18.9	366 \pm 42.6	190 \pm 16.8 ^{###}

One asterisk, two asterisks, and three asterisks compared to normal individuals

Two superscript number signs and three superscript number signs compared to regular blood transfusion HbE/ β -thalassemia patients

setting the gate around the platelet population, and then determining the percentage of platelets expressing CD62P and CD142 using the characteristic fluorescence profile of CD41a/CD62P or CD41a/CD142 in a bivariate plot.

Statistical analysis

Data were expressed as mean \pm SEM. The differences between subject groups were determined by the non-parametric Kruskal-Wallis test and p values <0.05 were considered statistically significant.

Results

Hematologic characteristics

Table 1 shows the hematological parameters and significant differences among subject groups recruited in this cross-sectional study. The anemic conditions and other

RBC indices typically found in thalassemic patients are reflected in the RT group. These findings were normalized after BMT. The one exception was the platelet counts, which were significantly lower in the BMT group than in the RT and normal groups.

Details of patients' disease background, transplantation characteristics, and frequencies of complications are given in Supplementary Table 1. Of the BMT group, 12 were patients with β -thalassemia major while 37 had β -thalassemia/HbE.

PS-externalizing RBC levels

Patients who had BMT had significantly lower percentages ($1.8 \pm 0.5\%$; range 0.2–60.9%) and absolute counts ($93,973 \pm 25,444$ cells/ μl ; range 10,440–869,960 cells/ μl) of RBCs expressing PS (Fig. 2a, b) in comparison to normal individuals ($0.2 \pm 0.01\%$; range 0.1–0.5%; 9846 ± 1323 cells/ μl ; range 4030–24,050 cells/ μl) and patients who received RT ($8.4 \pm 2.9\%$; range 0.2–60.9%; $395,524 \pm 116,259$ cells/ μl ; range 5340–2,362,920 cells/ μl).

Fig. 2 The percentages (a) and absolute counts (b) of PS-exposing RBCs in normal subjects, patients receiving regular transfusion (RT) or after bone marrow transplantation (BMT)

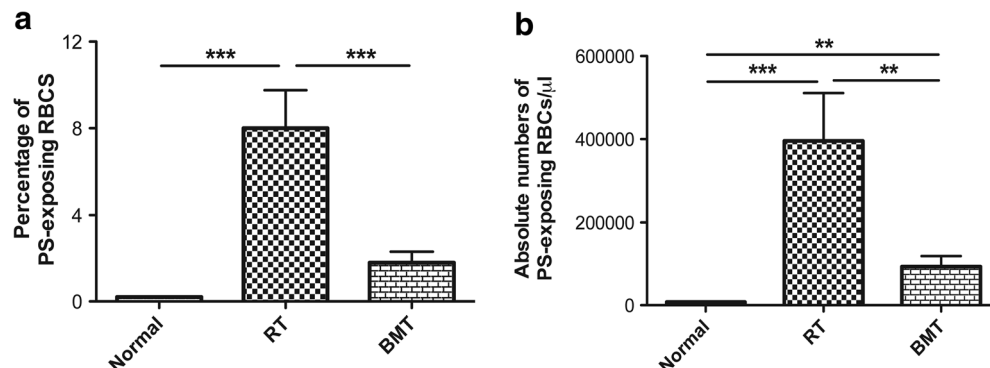
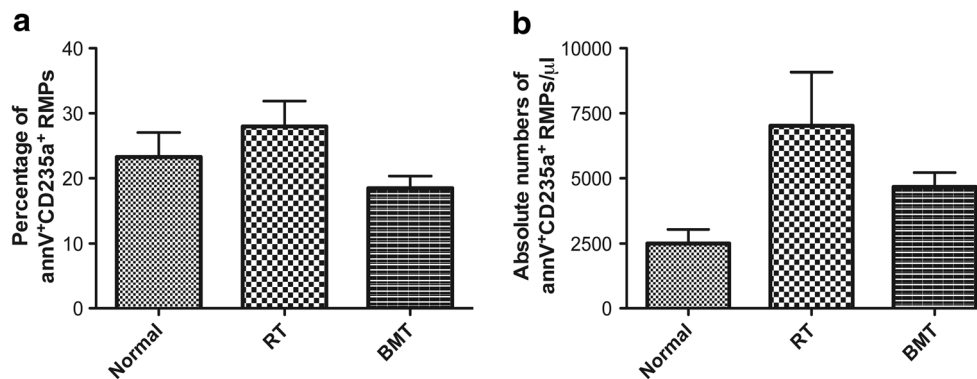


Fig. 3 The percentages (a) and absolute numbers (b) of circulating RMPs in normal subjects, patients receiving regular blood transfusion (RT) or after bone marrow transplantation (BMT)



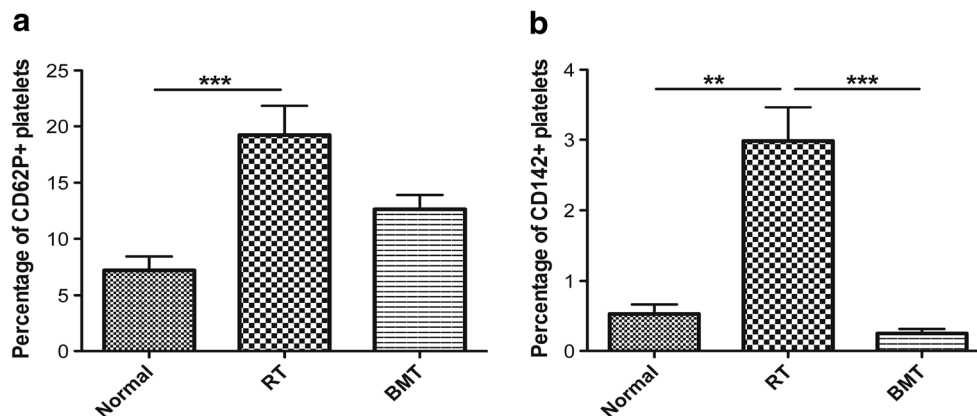
RMP levels

The percentages ($17.7 \pm 1.6\%$; range 1.1–43.9%) (Fig. 3a) and the absolute levels (4470 ± 488 events/ μ l; range 713–16,267 events/ μ l) (Fig. 3b) of RMPs in the BMT group, though not significant, tended to be less than in normal individuals ($23.6 \pm 2.3\%$; range 8.3–32.7%; 2506 ± 381 events/ μ l; range 380–5319 events/ μ l) and the RT group ($28.1 \pm 4.0\%$; range 0.9–62.2%; 6011 ± 1786 events/ μ l; range 299–25,298 events/ μ l).

Activated platelet levels

The percentages of procoagulant platelets in post-BMT thalassemia patients, defined by their expression of CD62P ($12.8 \pm 1.2\%$; range 2.6–42.63%) (Fig. 4a) and CD142 ($0.3 \pm 0.1\%$; range 0–2.3%) (Fig. 4b), were significantly less than in patients who received RT (CD62P: $17.9 \pm 2.3\%$; range 3.4–40.1% and CD142: $3.3 \pm 0.4\%$; range 0.1–7.1%), but did not differ significantly from percentages in normal individuals (CD62P: $7.3 \pm 1.3\%$; range 1.1–21.6% and CD142: $0.6 \pm 0.1\%$; range 0–1.3%).

Fig. 4 The percentages of platelets expressing CD62P (P-selectin) (a) and CD142 (tissue factor) (b) in normal subjects, patients receiving regular blood transfusion (RT) or after bone marrow transplantation (BMT)



Discussion

Allogeneic BMT provides an opportunity for hematopoietic reconstitution in vivo and the correction of the thalassemic phenotype. BMT for patients with β -thalassemia was first successfully performed over three decades ago [19]. The best cell source for BMT is a human leukocyte antigen-identical donor, thereby reducing the transplantation-related complications, including graft-versus host effect or immune rejection, and increasing the rates of disease-free survival [20]. However, BMT patients still require packed RBC transfusions to control the anemic condition, which results from the lack of fully functional erythropoiesis. When these infused RBCs age or are damaged, they externalize PS (from the inner membrane leaflet to the outer side) and are destroyed by the reticuloendothelial system, limiting their lifespan [21–23]. In addition, they can shed membranous MPs which also bear PS and other markers of their parental cells. In this context, they carry glycophorin A (CD235a) from RBCs, and so provide a means to access them by multi-parametric flow cytometry. These damaged RBCs and RMPs along with abnormal but intact RBCs are thought to be one of the etiologies of the thrombotic risk experienced by thalassemia patients.

In the present study, we could reliably measure the levels of PS-expressing RBCs and circulating RMPs in young patients with β -thalassemia major by flow cytometric analysis. Flow cytometric quantification of circulating blood cells and their components is a reliable and simple tool offering new insight into the association of RBC abnormality and the hypercoagulable state which occurs in thalassemia [14, 18]. Blood samples used in our investigations were collected before RBC transfusion to avoid confounding due to the RMPs from stored packed RBCs [16]. In our randomized study, we observed the significantly decreased percentages and total counts of PS-externalizing RBCs, shed RMPs, and activated platelets in a post-BMT patient group. Their levels were normalized to those found in normal individuals and significantly lower than the RT patient groups. This suggests that transplantation may correct, in part, the prothrombotic phenotype in β -thalassemia by means of reducing procoagulant risk factors. This is in concordance with the report of Sirachainan et al. that performing BMT in children with severe β -thalassemia can correct the abnormal levels of coagulation markers and anticoagulation factors toward normal baselines [24]. Tantawy et al. reported increased numbers of RMPs along with platelet-derived MPs in young thalassemia major patients, and positive correlations with vascular dysfunction, aortic stiffness, and pulmonary hypertension in those thalassemic patients [25]. The fact that RBC membrane vesiculates under oxidative stress conditions such as iron overload and the presence of excess unbound globin chains can explain why elevated PS exposure on RBCs and subsequent release of RMPs are found in β -thalassemic patients, particularly those received RT. Severely damaged RBCs are known to have an accumulation of unaffected globin chains in the cytoskeleton and a perturbation of lipid-protein membrane function, provoking the loss of RBC membrane asymmetry and the forming of MPs [6, 26, 27].

For the interpretation of PS-exposing RBC and RMP levels, looking at individual profile changes could be additionally helpful because each patient has different starting levels. In this study, there was no correlation between these two parameters and RBC counts. Therefore, the kinetic levels of RMPs and RBCs expressing PS cannot solely be explained by the different RBC counts. In addition, BMT patients also require platelet transfusion which implies that platelets are consumed in the coagulation activation resulting from endothelial cell damage [28], but platelet activation status has not been reported yet. In the present study, we demonstrated lower frequencies of platelets bearing P-selectin and tissue factor in patients after BMT. Regarding their role in the hypercoagulable state, we recently reported the prothrombotic potential of MPs on promoting platelet aggregation and neutrophil-platelet formation [29], which support the contention of further platelet activation by MPs. Furthermore, we have ruled out the possibility that different pre-conditioning regimens

might have impacted on our determined parameters, since no significant differences were found among BMT patients who received various conditioning regimens. This is in line with other reports in which there is no significant effect of pre-conditioning regimens on coagulation profiles nor on MP amounts [30, 31].

In summary, our present data show that BMT effectively decreases the concentrations of procoagulant circulating PS-exposing RBCs, their MPs, and procoagulant platelets compared to treatment with usual blood transfusion. Detection of MPs with other origins could be useful, supplementing the RMPs, broadening the potential diagnostic biomarkers for BMT.

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Authors' contributions PK, SV, EN performed the experiments, analyzed the data and wrote manuscript; SL, UA, NS supervised the project and analyzed the data; SH, KP designed the experiment, wrote, and edited the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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